Proteinases of betaretroviruses bind single-stranded nucleic acids through a novel interaction module, the G-patch

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Abstract Retroviral proteinases (PRs) are essential for retrovirus infectivity but the mechanism of their activity regulation is poorly understood. We investigated possible involvement in this process of the C-terminal domain (CTD) of betaretroviral PRs. We found that the presence of CTD attenuates proteolytic activity of Mason-Pfizer monkey virus PR, while it does not significantly affect the activity of mouse intracisternal A-particle retrovirus PR. However, both PRs bind single-stranded nucleic acids through their CTDs that contain a novel binding motif, the G-patch, whose function is dependent on a single conserved tyrosine residue. Oligonucleotide binding to both PRs does not inhibit their proteolytic activity.
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1. Introduction

Retroviruses express their aspartic proteinase (PR) as a part of polyprotein precursors. At the late stage of viral life cycle, PR is activated in the newly released immature virus particles and it cleaves viral polyproteins into individual functional components [1]. PR activity and its precise timing are essential for virus infectivity [2–4] and, in fact, the HIV-1 PR inhibitors are efficient virostatics (see [5–7] for recent detailed reviews). However, the mechanism of activation and activity regulation of retroviral PRs has not been fully understood to date.

Retroviral PRs are active only as homodimers. A current model for PR activation mechanism considers concentration-dependent dimerisation of polyprotein precursors to be the main factor contributing to PR activation in vivo. However, additional factors seem to play a role, such as the redox potential of the environment [8,9] or the cellular site of polyprotein assembly [10,11]. Whereas most retroviruses assemble and bud their capsids directly at the plasma membrane, a

retrovirus family known as betaretroviruses assemble in the cytosol. Their immature capsids are transported to the plasma membrane and their PR is activated only after the virus release from the cell [12]. This suggests that betaretroviral PR activity regulation might employ other mechanisms in addition to the concentration-dependent dimerisation of polyprotein precursors. Interestingly, betaretroviral PRs contain a conserved C-terminal extension of a hitherto unknown function (Fig. 1) that extends beyond the retroviral PR homology domain and might be involved in such a regulation mechanism.

A recent bioinformatic analysis of sequence data identified a previously unnoticed, conserved glycine-rich domain, named G-patch, in about 100 eukaryotic proteins and in betaretroviral polyproteins [13]. Importantly, the G-patch domain is located within the C-terminal extension of betaretroviral PRs. The G-patch domain has been proposed to have an RNA binding affinity, based on the fact that some of the eukaryotic G-patch containing proteins have been implicated in RNA processing [13], although no experimental data have been provided. Hence, there is an intriguing possibility that the activity of betaretroviral PRs might be regulated by nucleic acid ligands. In this report, we examine this hypothesis by investigating the role of the C-terminal domain (CTD) and its possible interaction with nucleic acids for regulation of beta-retroviral PR activity. For this purpose, we use two model PRs from related betaretroviruses: mouse intracisternal A-type particles endogenous retrovirus (MIA-14) and Mason-Pfizer monkey virus (MPMV).

2. Materials and methods

2.1. Oligonucleotides and chemicals
The following DNA oligonucleotides (ONs) were used for DNA cloning:
F1: 5′-GGGGTACCCATATGTTGGTTGTATCTTTAAATG-3′,
R1 (T7-terminator): 5′-GCTAGTTATTGCTCAGCGG-3′,
R2: 5′-GGGGATCCTCGAGCTATCAGGCGTTTTCATTGG-3′,
F3: 5′-GTAACTGCTTAAATGTTAGCC-3′,
R3: 5′-GGCTAACATTTAAGCAGTTAC-3′,
YS1F: 5′-GGCAAAGATGGGTTCGAAAGAAGGAAAAGGG-3′,
YS1R: 5′-CCCTTTTCCTTCTTTCGAACCCATCTTTGCC-3′,
YS2F: 5′-AGCCCAGGGGCTCCAGCCCGGGAAAAGGGTTAGG-3′, and

YS2R: 5′-CCTAACCCTTTTCCCGGGCTGGAGCCCTGGGCT-3′.

The following ONs were used for the electrophoretic mobility shift assay:

DNA ONs:

1: 5′-CACCTACACAAAGTGAGCTAGCTCTACGATCCGGT-GTTTCGTCCTTTCCACAAG-3,

2: 5′-CTGCCGACGCTCAACGAGACTCCCGAACAGGTTTG-CCA-3′,

3: 5′-GATTAGGAATTGTGGAACCAATAGAAAATATTTAC-3′,

4: 5′-GACCGCTCGGGCCCGAAGACAAGTACCGAG-3′,

5: 5′-TCGAGAGAGAGGATATTAAGAGTTGCAAAGATTTT-TTTTAATTCTGTTTC- 3′,

6: TGGCAAACCTGTTCGGGAGTCTCGTTGAGCGTCGGC-AG

RNA ONs:

7: 5′-GGACUAGCGGAGGCUAGUCC-3′, and

8: 5′-UCCUAGGUGAGUGAGACGUGUGCUU-3′.

All ONs were custom-synthesised on solid phase support and HPLC-purified. All other chemicals were of analytical grade.

2.2. Cloning

The DNA cloning was performed by standard methods [14]; site-directed mutagenesis was carried out using the Quick-change protocol (Stratagene) and the authenticity of all clones was confirmed by DNA sequencing.

MIA-14 PR variants were subcloned by PCR using the *Nco*I fragment of MIA-14 genome inserted in the pET16b expression vector as a template [15]. The full length MIA-14 PR expression construct was generated using the **F1** and **R1** primers that removed the 5′-end of PR precursor gene to encode mature MIA-14 PR [15]. The PCR product was cloned into pET22b expression vector (Novagen) between *Nde*I and *Nco*I sites to yield pET_MIAPR. The expression construct for the C-terminally truncated MIA-14 PR (MIA-14 PRΔCTD) was generated using the **F1** and **R2** primers that introduced stop codon after the Ala109 codon of mature MIA-14 PR (Fig. 1, symbol **1**). The resulting PCR product was inserted between *Nco*I and *Xho*I sites of pET22b expression vector to yield pET_MIAPRΔCTD.

The expression plasmid for the C-terminally truncated M-PMV PR (M-PMV PRΔCTD) was prepared by site-directed mutagenesis using pBP construct as a template [16,17] and the **F3** and **R3** primers that introduced a stop-codon at the 3′-end of M-PMV PRΔCTD gene. The corresponding M-PMV PRΔCTD protein spans amino acids 1–114 of the mature M-PMV PR (Fig. 1, symbol **2**) and it is identical to PR13 from that described in [17].

The MIA-14 PR Tyr126Ser mutant was generated by site-directed mutagenesis of the pET_MIAPR template using the **YS1F** and **YS1R** primers that changed the TAT (Tyr126) codon into TCG (Ser126) and introduced a unique BstBI restriction site. The MPMV PR Tyr121Ser mutant was constructed analogously, using pBP plasmid as a template

[16,17] and the **YS2F** and **YS2R** primers that changed the TAC (Tyr121) codon into TCC (Ser121).

2.3. Recombinant protein expression and purification

MIA-14 PR, MIA-14 PRY126S and MIA-14 PRΔCTD were expressed in *Escherichia coli* BL21(DE3) using the T7 promoter/T7 RNA polymerase transcription/translation system [18]. Suspension culture of transformed *E. coli* cells was grown at 37 °C in Luria–Bertani medium [14] supplemented with 100 μg/ml ampicillin. Recombinant protein expression was induced at culture OD$_{600}$ value of 0.8 by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. After 3 h of induction, bacteria were pelleted by centrifugation and frozen to −20 °C. To isolate recombinant proteins, bacterial cells were resuspended in a lysis buffer (50 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, pH 8.0) at circa 0.1 g cell paste per ml and desintegrated as described [15].

MIA-14 PR and MIA-14 PRY126S were isolated from the soluble fraction of the bacterial lysate. It was dialysed against 100 volumes of 50 mM Tris–HCl, 1 mM EDTA, 0.05% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol, pH 8.5, at 4 °C overnight and the dialysate was clarified by centrifugation. The PR variants were further purified by liquid chromatography using Q-Sepharose, SP-Sepharose Fast Flow and Superdex 75HR (all Pharmacia) columns and handled as described [15].

MIA-14 PRΔCTD was isolated from the insoluble portion of the induced bacterial lysate. The collected and washed [19] inclusion bodies were resuspended in 200 mM Tris–HCl, 100 mM sodium phosphate, 0.5% (v/v) 2-mercaptoethanol, and 20 mM EDTA, pH 7.0, at 0.3 g per ml and diluted 10-fold in freshly prepared 8 M urea heated to 40 °C. The suspension was stirred for 30 min and MIA-14 PRΔCTD was refolded by dialysis into 50 mM Tris–HCl, 4 mM EDTA, 8% (v/v) glycerol, and 0.1% (v/v) 2-mercaptoethanol, pH 7.5, at 4 °C and then into SP buffer (50 mM sodium acetate, 20 mM NaCl, and 0.05% mercaptoethanol, pH 5.0). The dialysate was clarified by centrifugation (30 000×g, 15 min, 4 °C) and purified on an SP-Sepharose Fast Flow column (Pharmacia) with elution by a linear gradient of 0–1 M NaCl in SP buffer. The eluted fractions containing MIA-14 PRΔCTD were pooled, dialysed against 50 mM sodium acetate, 200 mM NaCl, 2 mM EDTA, 10% (v/v) glycerol and 0.05% (v/v) 2-mercaptoethanol, pH 5.5, concentrated by ultrafiltration to circa 4 mg/ml and further purified on a Superdex 75HR gel filtration FPLC column (Pharmacia) in the same buffer.

All MPMV PR variants were expressed and purified as described [20]. All proteins were stored at −70 °C.

2.4. Activity assays and enzyme kinetics

Protein concentration was determined by the Coomassie dye binding assay (Bio-Rad) and enzyme activities were measured at 37 °C. The activity of MIA-14 PR variants was followed in a continuous spectrophotometric assay using synthetic chromogenic peptide substrate DSAYF(NO$_2$)VVS that mimics the N-terminal MIA-14 PR autoprocessing site [15]. Substrate cleavage was monitored at 305 nm. The


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MIA-14_PR   LVVSLN-DRPKLRLKINGKEFEGILDTGADKSIISTHWWPKAWPTTESSHSLQGL
MPMV_PR     WVQPITCQKPSLTLWLDDKMFTGLIDTGADVTIIKLEDWPPNWPITDTLTNLRGI
HIV-1_PR    -PQITLWQRPLVTIKIGGQLKEALLDTGADDTVLEEMNLPGRWKP----KMIGGI
            1........10........20........30........40........50....

MIA-14_PR   GYQSCPTISSVALTWESSEGQQGKFIPYVLP-LPVNLWGRDIMQHLGLILSNENA
MPMV_PR     GQSNNPKQSSKYLTWRDKENNSGLIKPFVIPNLPVNLWGRDLLSQMKIMMCSPND
HIV-1_PR    GGFIKVRQYDQILIEICGHKAIG--TVLVGP-TPVNIIGRNLLTQIGCTLNF---
            ....60........70........80........90........100.......

MIA-14_PR   PSGGYSTKAKNIMAKMGYKEGKGLGHQEQGRIEPISPNGNQDRQGLG-FP
MPMV_PR     -------IVTAQMLAQGYSPGKGLGKKENGILHPIPNQGQSNKKGFGNF-
HIV-1_PR    -------------------------------------------------
            111......120.......130.......140.......150........
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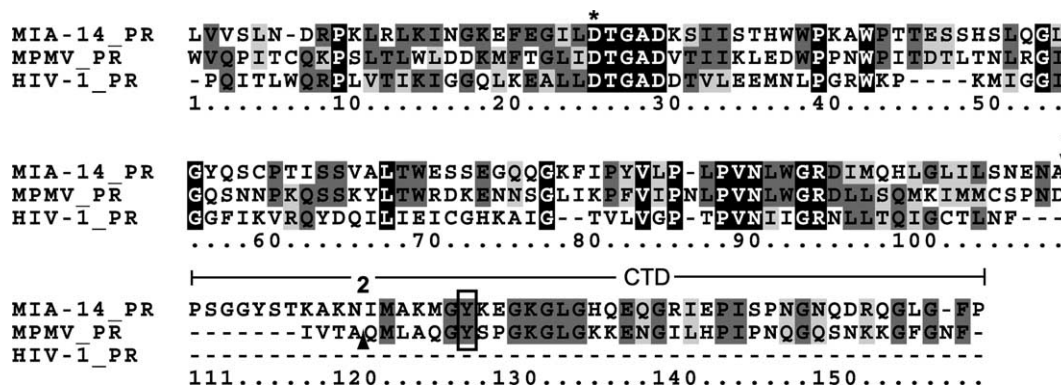
Fig. 1. The C-terminal extension of betaretroviral PRs extends beyond the retroviral PR homology domain and contains highly conserved glycine patches. Multiple protein sequence alignment of mature MIA-14 PR [15], MPMV PR (UniProt Accession No. P07570, residues 163–314) and HIV-1 PR (UniProt Accession No. 012834) was generated using the ClustalW software, version 1.82 [33]. Similar residues are shown on a light grey background, identical residues on a dark grey background, and the residues conserved among all three PRs are shown in white on a black background. The retroviral PR homology domain is defined by the sequence of lentiviral HIV-1 PR. Legend: * denotes the active site catalytic aspartate, **1** and **2** show the boundaries between the PR domain and the CTD of MIA-14 and MPMV PR, respectively. The conserved Tyr that was mutated to Ser in MIA-14 PRY126S and MPMV PRY121S is enclosed in a black box.

activity of MPMV PR and its variants was determined in a discontinuous assay using ATHQVYF(NO₂)VRKA peptide substrate whose cleavage was monitored by HPLC as described [20]. Kinetics constants of the Michaelis–Menten equation were determined from the initial reaction rates by non-linear regression fitting into the appropriate model equations using GraFit programme (Erithacus Software Ltd., Surrey, UK). The peptide substrates were synthesised on solid phase and purified by reversed-phase HPLC.

2.5. Electrophoretic mobility shift assay

All solutions used for the electrophoretic mobility shift assay (EMSA) experiments with RNA ONs were prepared using diethylpyrocarbonate-treated water. To facilitate detection, each DNA or RNA ON (50 ng) was radiolabelled by 5′-phosphorylation using T4 polynucleotide kinase (New England Biolabs) and 20 μCi of [γ-³²P]ATP (Amersham Biosciences). The labelled ONs were purified using MicroSpin G-25 columns (Amersham Biosciences) and immediately used for binding experiments. Labelled double-stranded DNA (dsDNA) was generated by annealing labelled ON **6** and a 3-fold molar excess of unlabelled ON **2** (that is complementary to ON **6**), heating the mixture to 96 °C for 2 min and cooling it down to room temperature within 2 h. In binding experiments, 2–7 nM labelled ON was incubated in the presence of 1–2 μM tested protein for 45 min at room temperature in 20 μL of reaction buffer (50 mM Tris–HCl, 4% (v/v) Ficoll 400, pH 8.0). Protein–ON complexes, which always display lower electrophoretic mobility than free ONs, were resolved by electrophoresis in a native 6% polyacrylamide gel in 0.5× TBE running buffer. Gels were dried and the radiolabelled material was detected by autoradiography.

3. Results

3.1. Cloning, expression and purification of the full-length and C-terminally truncated MIA-14 and MPMV PRs

We set out to study the possible role of the C-terminal extension in activity regulation of betaretroviral PRs. To this end we subcloned the C-terminally truncated MIA-14 and M-PMV PRs (designated MIA-14 PRΔCTD and MPMV PRΔCTD) into prokaryotic expression vectors based on their described N- and C-terminal autocleavage sites [15,20,21] and amino acid alignments of retroviral PRs (Fig. 1). All PRs were

heterologously overexpressed in *E. coli*. While MIA-14 PR variants were isolated from the soluble part of bacterial extracts in a folded and active state, MIA-14 PRΔCTD was expressed into bacterial inclusion bodies. The enzyme was isolated and in vitro refolded by urea-induced denaturation and gradual removal of the denaturant by dialysis. The efficiency of refolding was confirmed by gel-permeation chromatography where purified MIA-14 PRΔCTD migrated as a symmetric peak, which is characteristic for a folded protein (data not shown). The MIA-14 PR variants were further purified in a series of chromatographic steps to yield highly purified preparations (Fig. 2A). The MPMV PR and its mutants accumulated in inclusion bodies during expression in *E. coli* and they were isolated, in vitro refolded, and purified as described [20] (Fig. 2A). All protein preparations exhibited 280 nm/260 nm absorbance ratio in the range of 1.6–1.8, which is in accordance with the estimated values computed from the amino acid composition of PRs and absorption spectra of free amino acid chromophores Trp, Tyr and Phe [22], indicating the absence of significant amounts of endogenous nucleic acid contamination.

3.2. The influence of CTD on the proteolytic activity of MIA-14 and MPMV PRs

To determine the effect of betaretroviral PR CTD of proteolytic activity, we measured the Michaelis–Menten kinetics parameters and their pH dependence for both the wild type and truncated PR variants using purified enzymes and peptide substrates in vitro. As no tight-binding inhibitors that would allow the determination of accurate concentration of the active enzyme were available for either PR, we approximated k_{cat} by V_{max}/E_0 assuming the absence of any inactive enzyme species in our preparations.

The presence of the C-terminal extension in both MIA-14 and MPMV PRs does not influence the enzymes' pH optima that remain in the acidic range. The pH optimum of catalytic efficiency $V_{max}/(E_0 \cdot K_M)$ is at pH 5.3 for both the full-length and the C-terminally truncated MIA-14 PR (Fig. 2B). While
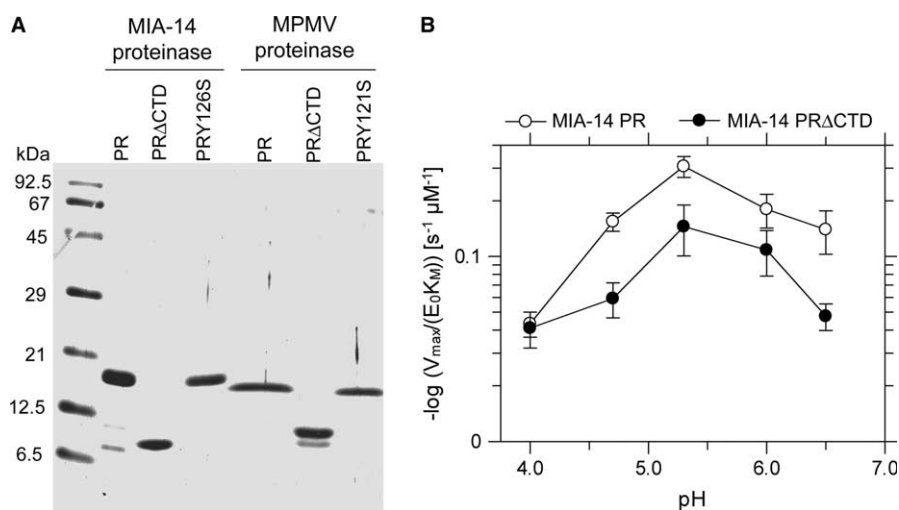

Fig. 2. (A) Purity of the recombinant MIA-14 and MPMV PR variants used in this study. Coomassie-stained 18% SDS–PAGE gel shows 0.2 μg of each enzyme per lane. Molecular weight markers are shown on the left. Partial autodegradation of some PR species within the CTD can be observed [15]. (B) The pH dependence of catalytic efficiency ($V_{max}/(E_0 \cdot K_M)$) of MIA-14 PR and MIA-14 PRΔCTD was determined using peptide substrate DSAYF(NO₂)VVS that mimics the N-terminal MIA-14 PR precursor autoprocessing site. Enzyme activity was determined as described in Section 2.4. The reaction buffer contained 100 mM buffer ion, 1 mM ethylenediaminetetraacetic acid (EDTA) and 2 M NaCl.

Table 1
Influence of the CTD on proteolytic activities of MIA-14 and MPMV PRs

Enzyme	K_M (μM)	V_{max}/E_0 (s^{-1})
MIA-14 PR	5.9 ± 0.6	1.81 ± 0.05
MIA-14 PRΔCTD	11.3 ± 2.5	1.64 ± 0.14
MPMV PR	36 ± 5.1	0.24 ± 0.04
MPMV PRΔCTD	386 ± 42	3.2 ± 0.30

The activity of MIA-14 PR and MIA-14 PRΔCTD was determined spectrophotometrically at 37 °C in a continuous assay, using peptide substrate DSAYF(NO$_2$)VVS that mimics the N-terminal MIA-14 PR precursor autoprocessing site [15]. The reaction buffer of pH 5.3 contained 100 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 M NaCl and 0.05% (v/v) 2-mercaptoethanol. The activity of MPMV PR and MPMV PRΔCTD was determined by a discontinuous assay using ATHQVYF(NO$_2$)VRKA peptide substrate whose cleavage was monitored by HPLC, as described [20]. The reaction buffer of pH 5.3 contained 50 mM sodium acetate, 1 mM EDTA, 0.3 M NaCl and 0.05% (v/v) 2-mercaptoethanol. The initial reaction velocities were fitted into the Michaelis–Menten equation by non-linear regression. The resulting kinetic constants are expressed as mean values ± standard error of the mean.

the Michaelis constant (K_M) of MIA-14 PR and MIA-14 PRΔCTD shows only slight and similar increase with pH, the V_{max}/E_0 is strongly pH-dependent with a maximum at pH 5.3 (data not shown). The differences in K_M or V_{max}/E_0 between MIA-14 PR and MIA-14 PRΔCTD are rather marginal, maximum 2-fold within the studied pH interval. On the other hand, MPMV PR variants display greater differences in activity. Even though the catalytic efficiencies $V_{max}/(E_0 \cdot K_M)$ of MPMV PR and MPMV PRΔCTD are almost identical, MPMV PRΔCTD displays about 10-fold higher K_M and V_{max}/E_0 than MPMV PR. Comparison of kinetic parameters of MIA-14 and MPMV PR variants at their pH optima is summarised in Table 1.

3.3. MIA-14 and MPMV-PRs bind single-stranded (ss) nucleic acids through interactions with their CTDs

To analyse whether the putative nucleic acid binding domain, the G-patch [13], predicted to be present within the C-terminal extension of betaretroviral PRs could bind nucleic acids, we performed electrophoretic mobility shift assay (EMSA) using purified PR variants and five DNA and two RNA ONs varying in length and GC content. In all cases, we observed ON binding only to full-length PRs and not to their C-terminally truncated variants, demonstrating that nucleic acid binding to both betaretroviral PRs is mediated by their CTDs. Both MIA-14 and MPMV PRs bind DNA ONs **1–5** whose lengths range from 30 to 54 bases, and GC content from approximately 30–70% with comparable efficiency, showing the absence of a clear sequence specificity of binding to ssDNA (see Fig. 3A and B for ON **1** and Fig. 3C for ON **6** as examples). However, while MIA-14 PR binds both RNA ONs **7** and **8**, the MPMV PR binds only ON **8**. Furthermore, the MIA-14 PR forms three complexes with RNA ON **8** that differ in electrophoretic mobility, while MPMV PR forms only the lowest mobility complex with ON **8**. This might be caused by different binding stoichiometry of PR–ON complexes and/or the presence of various conformational isomers of ON **8**. Both observations together suggest that the two G-patch domains might have distinct binding specificities.

Next, we directly compared the affinities of PR binding to ssDNA and dsDNA. In this experiment, the addition of a 3-fold molar excess of unlabelled ON **6** over the labelled ON **6** partially decreases the intensity of the ON **6**–MIA-14 PR complex band but does not abolish it, while the addition of 3-fold molar excess of unlabelled complementary ON **2** over labelled ON **6** leads to only the dsDNA being visible on the EMSA gel and no ON **6**–PR complexes being observable (Fig. 3C).
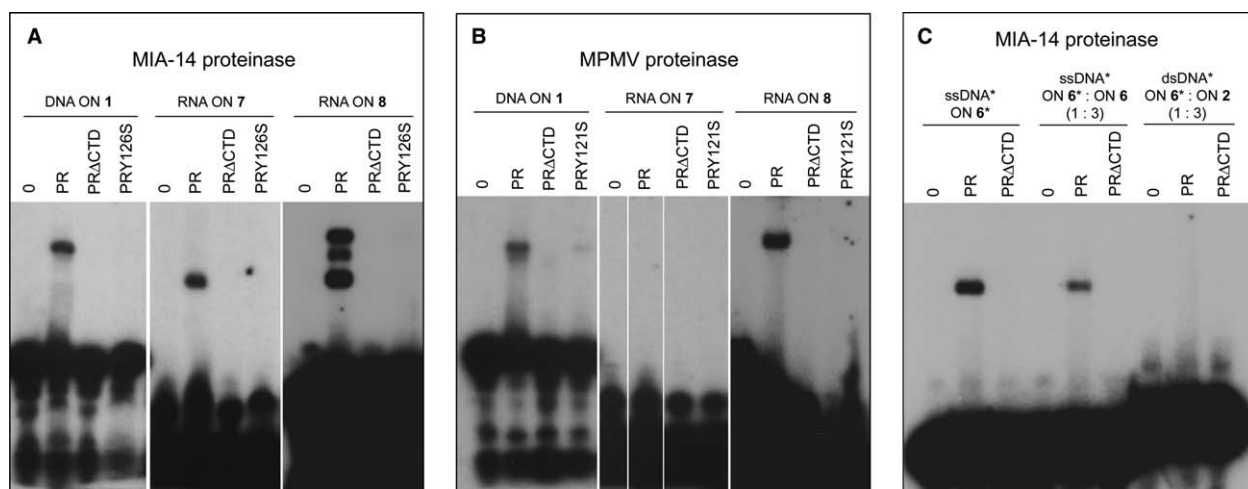

Fig. 3. MIA-14 and MPMV-PRs bind ssDNA and RNA through their C-terminal extension. (A) Autoradiogramme of the electrophoretic mobility shift assay (EMSA) polyacrylamide gel comparing the binding affinity of the full-length MIA-14 PR, its C-terminally truncated mutant (PRΔCTD) and the PR variant mutated in the conserved tyrosine within CTD (PRY126S) to DNA ON **1** and RNA ONs **7** and **8**. (B) EMSA gel showing identical analysis for the MPMV PR. The protein–ON complexes that always have lower electrophoretic mobility than free ONs can be observed as separate bands in the top part of the gels. The EMSA experiments were carried out as described in Section 2.5. (C) Comparison of binding affinity of MIA-14 PR to ss and ds DNA. Radioactively labelled (*) dsDNA was generated by labelling ssDNA ON **6** and adding 3-fold excess of unlabelled ON **2** that is exactly complementary to ON **6**. The addition of a 3-fold molar excess of unlabelled ON **6** over the labelled ON **6** partially decreases the intensity of the ON **6**–PR complex band but does not abolish it, while the addition of 3-fold molar excess of unlabelled complementary ON **2** to the labelled ON **6** leads to only dsDNA being visible on the EMSA gel and no PR–ON **6** complexes being observable. Notice that dsDNA has a slightly lower electrophoretic mobility than ssDNA.

To prove that the nucleic acid binding affinity of the CTD of MIA-14 PR is a specific property of the domain structure, we selected a highly conserved aromatic residue that is present in all analysed G-patch domains [13], assuming that it might be important for the domain function, and mutated it to Ser. We have prepared recombinant MIA-14 PRY126S and MPMV PRY121S and tested their nucleic acid binding properties using EMSA. While the proteolytic activity of both mutant PRs was not affected, we could not detect any significant binding affinity of MIA-14 PRY126S and MPMV PRY121S to nucleic acids (Fig. 3A and B). This confirms that the binding of betaretroviral PR to nucleic acids is mediated specifically by the G-patch domain, and that its conserved Tyr residue is important for binding specificity or for maintaining domain structure.

3.4. Nucleic acid binding to MIA-14 and MPMV PRs does not significantly affect their proteolytic activity

To analyse whether nucleic acid binding to the CTD of MIA-14 PR could modulate proteolytic activity of the PR domain, we determined the influence of a 5.4-fold molar excess of ON **2** on the activity of MIA-14 and MPMV PR, PRΔCTD, and PRY $\rightarrow$ S. In the presence of ON **2**, MIA-14 PR activity increased to 148%, MIA-14 PRΔCTD to 118% and MIA-14 PRY126S only to 103% relative to the activity of the respective enzyme in the absence of ON **2** (Fig. 4). On the other hand, the activity of MPMV PR, MPMV PRΔCTD, and MPMV PRY121S was not influenced by ON **2** binding at all. The slight activatory effect of ON **2** on MIA-14 PRΔCTD might be non-specific and due to a lower stability of MIA-14 PRΔCTD relative to MIA-14 PR, since we observed a similar effect caused by the polyanionic polymer heparin (not shown). The activity of PRY $\rightarrow$ S mutants that do not bind any ONs in our EMSA assays is practically not affected by the presence of ON **2**. In conclusion, a randomly chosen DNA ON **2** that binds to MIA-14 and MPMV PRs (Fig. 3C) does not have significant influence on their activities.

4. Discussion

The mechanism of activity regulation of retroviral PRs is still poorly understood. Betaretroviruses are excellent models for studying PR activation because the processes of their polyprotein assembly and PR activation are spatially and temporally separated. In those particular examples of beta-retroviruses that we studied, the MPMV virus particles form in the cytoplasm and are proteolytically processed only after virus budding from the cell, while MIA-14 endogenous retrovirus particles assemble and bud through the endoplasmic reticulum membrane, accumulate inside the ER and are never proteolytically processed there. Betaretroviral PRs, unlike the lentiviral PRs, might utilise an additional control mechanism to delay their activation until viral budding occurs. Interestingly, betaretroviral PRs contain an ~50 amino acid long C-terminal extension that is not present in other retroviral PRs. In this report, we investigated the potential role of the C-terminal extension in regulation of betaretroviral PR activity.

The "economy" of organisation of retroviral genomes suggests that it would be unlikely that a conserved protein domain would not serve any purpose. Several PRs of betaretroviruses and related endogenous retroviruses that contain the C-terminal extension, such as MPMV, MIA-14, human endogenous retrovirus K10 (HERV K10), and rabbit endogenous retrovirus H [23], formerly known as human retrovirus 5 [24], have been shown to undergo sequential processing from the C-terminus both in vitro [15,21,23,25] and in vivo [20]. However, the functional importance of this process has not been elucidated. The C-terminally truncated form of HERV K10 PR was shown to have about 20-fold higher activity than the full-length form and the authors thus hypothesised that PR C-terminal extension might act as an intramolecular inhibitor [25]. In our experiments, the C-terminally truncated MIA-14 PR has about half of the activity of the full-length MIA-14 PR, which would contradict that hypothesis, but PR activity of an endogenous retrovirus might not be relevant, since endogenous retrovirus polyproteins usually do not undergo proteolytic processing inside the cells. On the other hand, the observations regarding MPMV PR and MPMV PRΔCTD are in line with the above hypothesis. Although the catalytic efficiencies $V_{max}/(E_0 \cdot K_M)$ of MPMV PR and MPMV PRΔCTD are almost identical, the C-terminally truncated variant MPMV PRΔCTD displays about 10-fold higher K_M and V_{max}/E_0 than the full-length MPMV PR. In this respect, it should be remembered that within the virion where PR substrate concentration is high, about 1 mM [26], PR will be substrate-saturated and it is then the V_{max}/E_0 that determines its catalytic power. Hence, in the case of MPMV PR, the presence of its C-terminal extension may indeed act to down-modulate PR activity in cis. However, this is unlikely to be its only role since less than half of MPMV PR is C-terminally processed in the virions [20].

A domain homologous to betaretroviral PR CTD has been recently identified in over 100 eukaryotic proteins [13]. It contains several conserved glycine residues and was therefore named 'G-patch'. It was suggested that this domain mediates RNA-protein interactions, based on an indirect observation
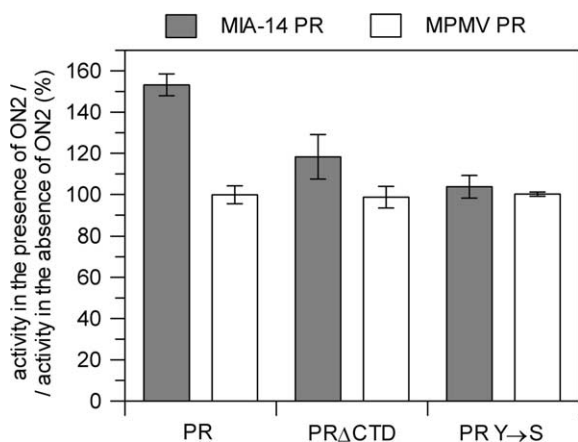

Fig. 4. The influence of nucleic acid binding on MIA-14 PR and MPMV PR proteolytic activity. The activity of MIA-14 PR variants (grey bars) and MPMV PR variants (white bars) was determined in the presence of 5.4-fold molar excess of ON **2** and expressed relative to the activity of the respective enzyme in the absence of ON **2**. Reaction mixtures contained 250 nM enzyme, 1.35 μM ON **2**, 100 mM sodium acetate, pH 5.3, 100 mM NaCl and 0.05% (v/v) 2-mercaptoethanol. After 20 min preincubation of the mixtures, proteolytic reaction was started by the addition of 250 μM substrate peptide ATH-QVYF(NO$_2$)VRKA and allowed to continue for 10 min. Cleavage products were separated and quantified by reversed-phase HPLC as described [17]. Each value was determined in triplicates and the results are expressed as the mean value ± standard error of the mean.

that many of the proteins in which it is present are implicated in RNA processing. In this report, we provide direct proof that the G-patch domain located within the C-terminal extension of betaretrovirus PRs efficiently binds ss nucleic acids. We found that its binding specificity might differ between betaretroviruses and is strongly dependent on a conserved aromatic residue (mostly Tyr) that might be involved in critical interactions with nucleic acid bases [27].

The possibility that a PR function could be regulated by interaction with a nucleic acid is not unique in the literature. It has been observed previously that the cleavage of the p15NC protein in HIV-1 by the HIV-1 PR is activated by viral RNA or RNA ON binding to p15NC in vitro [28]. However, the mutations in the RNA binding domain of p15NC introduced into an infectious clone of HIV-1 only slightly delayed p15NC processing without significantly compromising virus morphogenesis [29]. In another, perhaps more relevant, example, the coxsackievirus B3 PR 3C was shown to efficiently bind an RNA element within the 5' non-translated region of the viral RNA [30], which serves for the assembly of protein complexes necessary for the initiation of cap-independent translation and positive-strand RNA synthesis. However, the role of proteolytic activity of the 3C PR in these protein complexes is unclear at present [31].

We observed that the binding of a short ON to the CTD of MIA-14 and MPMV PRs does not have any significant activatory or inhibitory effects on the proteolytic activity (Fig. 4). Thus, it appears that in a mature betaretroviral PR the two domains, the PR domain and the G-patch, are functionally independent. We thus hypothesise that: (1) a long nucleic acid molecule binding to the CTD of the PR monomer, that is still a part of the viral polyprotein precursor, might interfere with PR precursor domain dimerisation and subsequent PR activation in virions and (2) after the ultimate PR activation and virion maturation, the C-terminal extension of a mature betaretroviral PR might target PR preferentially to its substrates that are associated with a nucleic acid. In support of the second point, it should be pointed out that during translation of MPMV polyprotein precursors, viral enzymes reverse transcriptase (RT) and integrase are produced by a –1 ribosomal frameshift at the 3' end of PR open reading frame [32]. The resulting GagProPol polyprotein contains the G-patch domain inserted between the PR and RT domains. It is possible that proteolytic processing of GagProPol generates RT containing the G-patch domain at its N-terminus. The presence of a G-patch in both PR and RT might allow co-targeting of both enzymes to an RNA domain and thus would facilitate a "directed" proteolytic processing of RT or other associated proteins. It remains to be shown whether any of these two points are valid in vivo, i.e., whether the G-patch domain might be important for the timing and extent of proteolytic processing of MPMV polyproteins and/or activity or specificity of MPMV RT.

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